での教室におけるMUC1-CTL療法を併用しなかった1年生存率約60%に比べ良好な成績を得ることができた。

3）MUC1-DC + MUC1-CTL療法

① DC

DCは強い抗原提示能力をもつ細胞として着目され，外部からの異物に対して抗原特異的免疫応答の要として働く細胞である。癌免疫療法でDCを用いることは，より効果的で強力な腫瘍特異的免疫応答を誘導する上で有効であり，われわれも従来用いてきたMUC1-CTL療法を発展させ，現在，MUC1-CTL療法にMUC1-DCを併用した細胞療法を行っている。

② 方　法

膵癌患者の末梢血単核球からleukapheresisにより付着細胞と浮遊細胞に分離し，前者はIL-4，GM-CSF，腫瘍壊死因子（tumor necrotizing factorα：TNFα）の存在下に培養を行い成熟DCを誘導し，これに癌抗原ペプチドであるMUC1ペプチドを成熟前にパルスし，MUC1ペプチドを細胞表面に提示したDC（MUC1-DC）を誘導，その後皮内投与する。後者はYPK-1と混合培養し，低用量のIL-2の刺激を行いMUC1-CTLを誘導し，静脈内投与する（図1）。これを10日ごとに繰り返し行う。

③ 成　績

再発，切除不能膵癌11例に対して，MUC1-DCとMUC1-CTLの併用療法を繰り返し施行した結果，2例でNC，1例CRであった。また，全例副作用の出現なく，外来で安全に施行できた。

④ 問題点

本療法は切除不能膵癌に対してある程度の臨床効果を期待できるものと思われる。しかし，担癌状態ではT細胞機能が全般的に低下しているため，その効果には限界があることを認めざるを得ない。また，手技がやや煩雑である上，多大な費用がかかる（1回につき十数万円）ことも，問題点の1つと言えよう。

4．癌ペプチドワクチン療法

1）方　法

癌ワクチン療法として，われわれは約9個のアミノ酸からなる

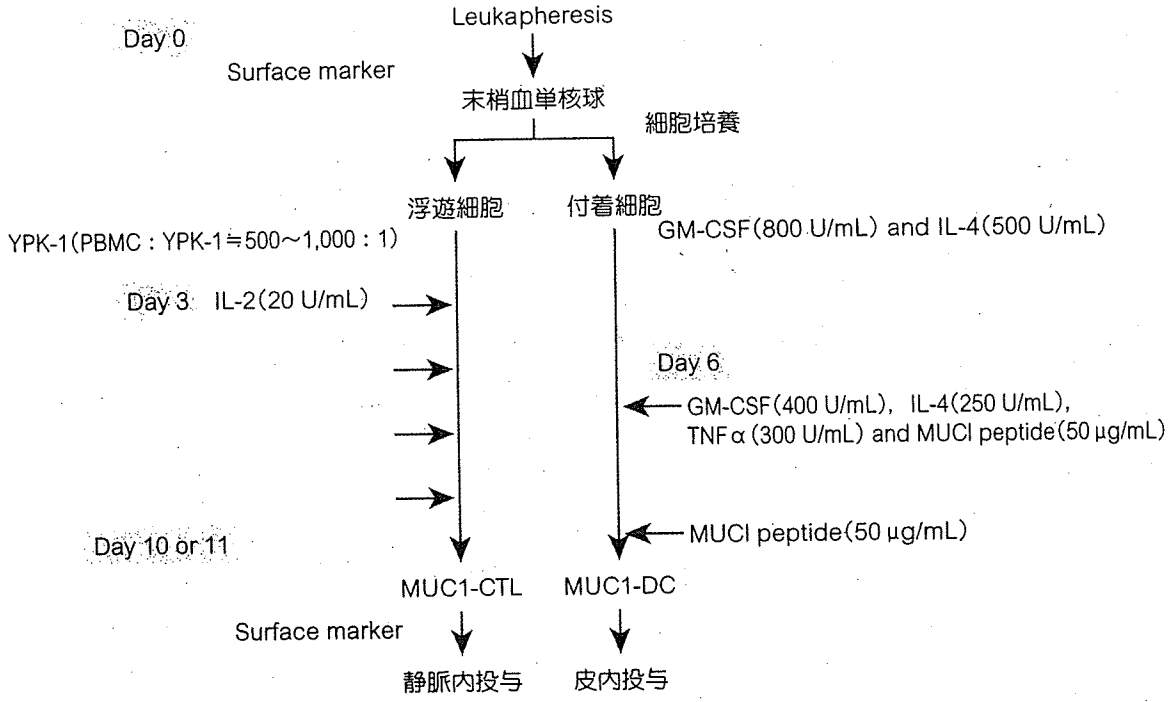

図1 MUC1 認識 CTL + MUC1 ペプチドパルス DC 併用療法

膵癌患者の末梢血単核球から leukapheresis により付着細胞と浮遊細胞に分離し，前者は IL-4，GM-CSF，TNF-α の存在下に培養を行い成熟 DC を誘導し，これに癌抗原ペプチドである MUC1 ペプチドを成熟前にパルスし，MUC1 ペプチドを細胞表面に提示した DC（MUC1-DC）を誘導，その後皮内投与する。後者は YPK-1 と混合培養し，低用量の IL-2 の刺激を行い MUC1-CTL を誘導し，静脈内投与する。これを 10 日ごとに繰り返し行う。

ペプチドを用いている。ペプチドは十数種類あるため，投与前にペプチドを選択する必要がある。そのため患者末梢血中にワクチン候補ペプチドに反応する CTL 前駆細胞が存在するか否かを調べ，CTL 前駆細胞の存在するペプチドを最高 4 種類まで同時に投与する。投与は 2 週間に一度，大腿部皮下に注射する。

2）成　績

再発・切除不能膵癌 10 例に本療法を施行したところ，PD9 例，NC1 例であった。NC の 1 例は，2 週間隔で 18 回以上の投与を行ったが，画像上明らかな再発病変は指摘できず，腫瘍マーカーも漸減し，長期 NC を得られている。安全性評価として，grade Ⅱ以下の局所発赤・腫脹，発熱が見られるのみで重篤な副作用は認めなかった。

5．化学療法

膵癌はほとんどが腺癌であるため，腺癌に対し抗腫瘍効果を有する抗癌剤が選択される。投与経路としては全身投与（静脈内投与）が一般的であるが，高い組織内濃度を得ることによる治療効果の増強を期待して，抗癌剤を動脈内に投与することもある。

従来 5-FU が，他の薬剤と同等かそれ以上の治療効果を有し，副作用も軽度であることから膵癌に対し最も頻用されてきた。また，最近ゲムシタビンが 5-FU より優れていると報告され[5]，注目されている。

多剤併用療法では 5-FU を中心に，種々の療法が試みられているが，進行膵癌に対する標準的な併用療法は確立していない。

おわりに

膵癌に対して，化学療法をはじめ従来の治療法には限界があることは明らかであり，新たな治療法の開発が待たれている。われわれが行ってきた免疫療法は副作用なく施行でき，切除例における肝転移抑制など効果を認めている。免疫療法は膵癌に対する新たな治療法のひとつになると期待される。

（長島由紀子，山本光太郎，矢原　昇，河岡　徹，岡　正朗）

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1. 癌ワクチン療法

はじめに

ヒト癌免疫療法は，1990年代以降科学的根拠に裏付けられた新時代に入った。免疫学と細胞生物学，分子生物学の進歩が種々の悪性腫瘍に発現する腫瘍抗原の分子レベルでの解明，すなわち腫瘍拒絶抗原ペプチドの同定を可能とし，他方では，強力な抗原提示細胞（antigen presenting cell：APC）である樹状細胞（dendritic cell：DC）の特性解析が進み，その *in vitro* 培養法が確立されたからである。これらの進歩を背景に癌免疫療法の新戦略であるワクチン療法の臨床応用が開始された。

本項では癌ワクチン療法の理論と実際，現況について概説し，今後の方向性を展望する。

1. 癌免疫療法の分類

癌免疫療法は能動免疫療法と受動免疫療法の2つに大別することが可能である。そしてその各々において，癌抗原を標的とした特異的免疫療法と，生体の免疫能全般を賦活することにより癌の退縮を期待する非特異的免疫療法が存在する。

また，リンパ球，単球等の免疫細胞を一旦体外に取り出した後に培養，増殖させ，抗腫瘍性効果細胞に変換した後に再び生体に投与する治療法は，細胞免疫療法と総称される。この治療法は受動免疫療法においては従来からその主体をなしてきたが，強力な抗原提示細胞であるDCの培養技術の進歩により，近年では能動免疫療法（ワクチン療法）における著しい進歩がもたらされている。

その他，IFNやIL-2等のサイトカイン製剤を用いたサイトカイン療法や，免疫賦活剤（biological response modifier〔BRM〕製剤）を用いたBRM療法が存在する。BRM製剤の中でもピシバニール®（OK-432）やBCGなどの菌体由来製剤は強力なサイトカイン誘導能を有し，その投与は生体の免疫能を非特異的に賦活する。感染とは直接関係はないが，ある意味感染免疫を応用した非特異的ワ

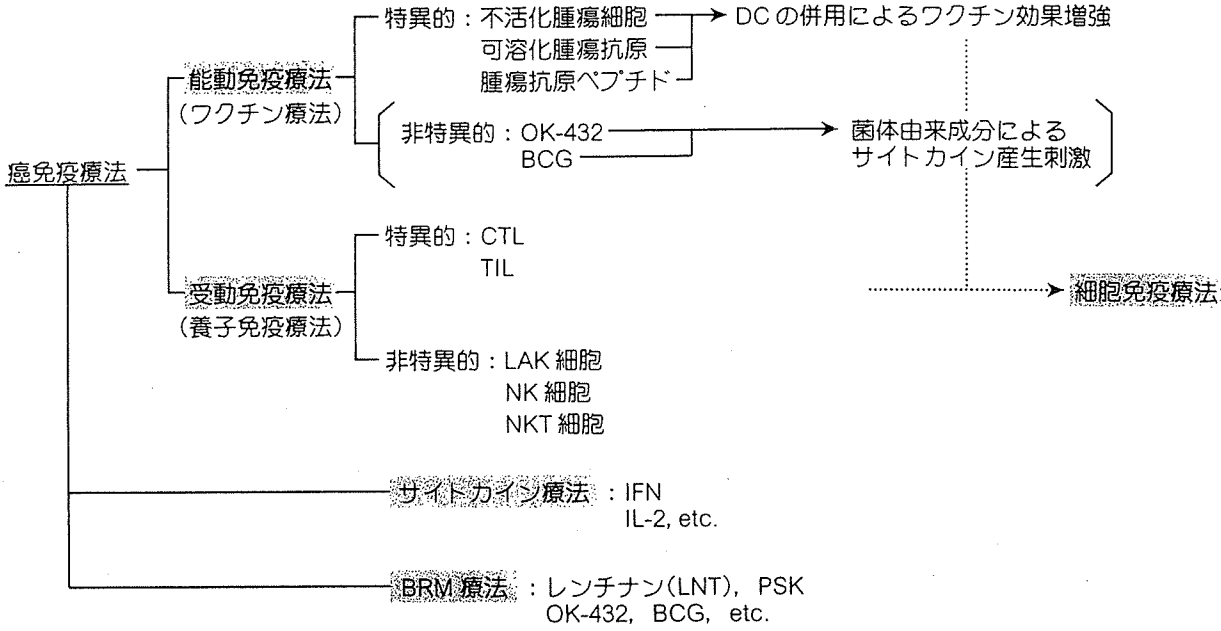

図1 癌免疫療法の分類

癌免疫療法は，能動免疫療法と受動免疫療法の2つに大別され，また，サイトカイン療法やBRM療法が存在する。

クチンとして，癌治療における位置づけが可能であろう（図1）。

2．癌ワクチン療法の理論と実際

癌ワクチン療法とは，癌に対する特異的能動免疫療法の別名である。ワクチン療法は元来，生体の免疫反応を利用して感染症の予防を行うことを主目的に発展してきた治療法であるが，その手法が癌治療にも応用されるようになった。すなわち，患者の癌に由来する何らかの抗原を接種することにより，癌に対する特異的T細胞免疫応答を惹起し，癌の再発予防や退縮を期待するものである（図2）。

癌抗原の科学的本体が不明であった90年代以前には，不活化した腫瘍細胞自体を用いたワクチン療法の臨床試験が，悪性黒色腫（メラノーマ）や腎癌，大腸癌に対して試みられたが，十分な効果は得られなかった[1]。それが90年代以降のメラノーマ抗原を中心とした数多くの$CD8^+$T細胞認識腫瘍抗原の分子レベルでの解明と，DCに関する研究の進展を契機として，急激な進歩を遂げてきている。

T細胞受容体は癌細胞表面上の癌抗原・主要組織適合遺伝子複

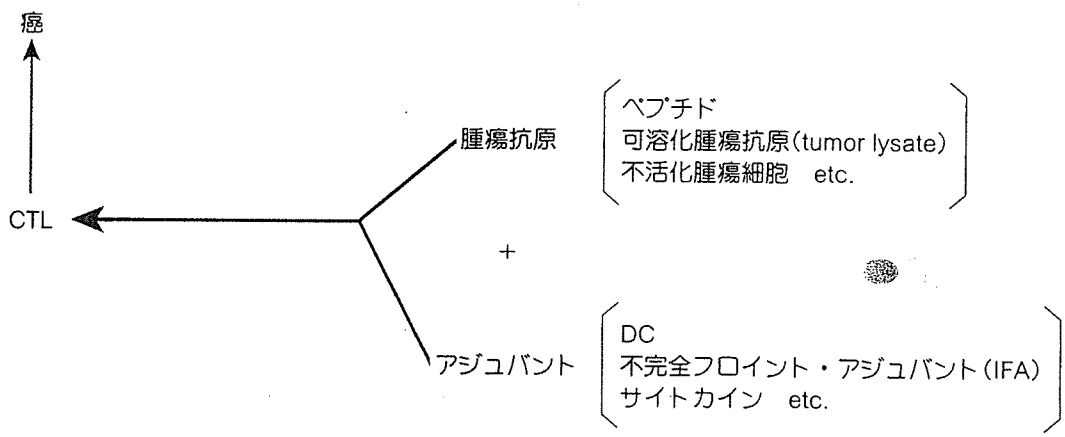

図2　癌ワクチン療法

患者の癌に由来する抗原を接種することにより，癌に対する特異的T細胞免疫応答を惹起し，癌の再発予防や退縮を期待する。

合体（major histocompatibility complex antigen：MHC）を抗原特異的に認識するが，T細胞が認識する抗原の本体はわずか9～15個ほどのアミノ酸残基からなるペプチドである。$CD8^+$T細胞はMHCクラス1分子と拘束性があり，その認識抗原ペプチド（$CD8^+$腫瘍特異的細胞傷害性T細胞〔cytotoxic T lymphocyte：CTL〕誘導性エピトープペプチド）が腫瘍拒絶抗原として，機能的DNA発現クローニング法を中心とした様々な手法を用いて数多く同定されてきた[2]。その代表的なものには，精巣と癌細胞にだけ発現する抗原（cancer/testis antigen）であるMAGEや，組織分化抗原（differentiation antigen）であるMART-1やgp100，癌に高発現する抗原（over expressed self antigen）であるCEAやHer2/neu等がある。これらの多くは，白人に多いHLA-A2拘束性あるいは日本人に多いHLA-A24拘束性ペプチドとして同定されている[3]。

しかし，いくら癌抗原の本質として分子レベルで同定された癌抗原ペプチドであるとはいえ，元来低免疫原性であるためその免疫効果は弱い。よって，実地臨床においては免疫効率を増強する

ための様々な補薬(アジュバント)が併用されることが多い。簡便であるため，その入手，調達が可能であれば，不完全フロイント・アジュバント（incomplete Freund adjuvant：IFA）やIFN，IL-2等のサイトカイン製剤が用いられることが多いが（欧米の臨床試験においては，IL-12やGM-CSFも用いられている），アジュバントとして間違いなく最も有能なものの1つは，ネーチャー・アジュバントと別称されるDCである。

DCはMHC分子や共刺激分子（CD80〔B7-1〕，CD86〔B7-2〕）を強発現し，抗原未感作のナイーブT細胞をも刺激することのできる，生体内で唯一の最も強力なAPCである。DCは流血中の単核細胞中に極微量の0.1%前後しか存在せず，その純化，増幅も困難であったため，その臨床応用が妨げられてきた。しかし，1994年にSallustoらにより，末梢血単球からGM-CSFとIL-4の存在下に比較的容易にDCを分化，誘導できることが明らかにされ[4]，腫瘍抗原ペプチドとDCの組み合わせによる癌ワクチン療法の臨床応用が開始された。9～15個のアミノ酸から構成されるペプチドをパルスされたDCは，MHCクラス1分子の溝にそれを提示し，強発現している共刺激分子とともに腫瘍特異的$CD8^+$ CTLを効率的に刺激，誘導する。担癌宿主の細胞性免疫機構が正常に作動すれば，生体に投与されたDCは近傍のリンパ節に遊走し，そこでCTLの誘導を促し，リンパ流に乗ったCTLは体内を一巡して腫瘍を攻撃し，消退へと導く。これが，癌ワクチン療法の理論である。

3．癌ワクチン療法の現況

欧米(白人)ではメラノーマ患者が多く，なおかつメラノーマが抗原性が高く免疫療法が奏効しやすいという特性を有しているため，メラノーマに対するワクチン療法の臨床試験が積極的に展開されてきた。実際に有効症例が数多く報告されており，化学療法と腫瘍縮小効果，生存期間を比較検討するための第3相試験も開始されている[5),6)]。

一方，本邦においてはメラノーマ患者が少ないため，消化器癌に対する臨床試験が多く行われているのが特徴である。1997年以降，大学病院を中心とした多くの医療研究機関，その中でもとりわけ外科系の診療科において，ワクチン療法が試みられてきてい

表1　本邦における固形癌に対するワクチン療法の臨床試験

施　設	対象疾患	使用腫瘍抗原	併用アジュバント
九州大学生体防御医学研究所	消化器癌	ペプチド（MAGE）	DC
和歌山県立医科大学	消化器癌	ペプチド（CEA）	DC
京都府立医科大学	消化器癌，肺癌	ペプチド（CEA，MAGE）	DC，（IFN-α，OK-432）
	メラノーマ	ペプチド（MAGE，tyrosinase），tumor lysate	DC
国立がんセンター中央病院	前立腺癌	融合蛋白（GM-CSF/PAP）	DC
	メラノーマ	ペプチド（MAGE，tyrosinase，gp100）	DC
山梨医科大学	胃　癌	ペプチド（Her2/neu）	DC
東京女子医科大学	消化器癌	tumor lysate，ペプチド（CEA，Her2/neu，Muc1）	DC
慶応義塾大学	膀胱癌	ペプチド（MAGE）	DC
滋賀医科大学	乳癌，肺癌	ペプチド（Muc1）	DC
九州大学（腫瘍制御）	消化器癌	tumor lysate，ペプチド（CEA）	DC，OK-432
岩手医科大学	消化器癌，乳癌	tumor lysate，ペプチド（CEA，Muc1）	DC
東京大学医科学研究所	転移性皮下腫瘍	なし（腫瘍局所放射線照射）	DC，IL-2
産業医科大学	肺　癌	ペプチド（MAGE）	OK-432
	消化器癌，肺癌	ペプチド（SART，CypB，Lck，ART，etc.）	IFA*
久留米大学	泌尿器科癌	ペプチド（同上）	IFA*
	婦人科癌 etc.	ペプチド（同上）	IFA*
北海道大学	消化器癌	ペプチド（同上）	IFA*
山口大学	膵　癌	ペプチド（同上，Muc1）	IFA*，DC
東京慈恵会医科大学	脳腫瘍，消化器癌	融合細胞（腫瘍/DC）	
帝京大学	消化器癌	融合細胞（腫瘍/DC）	

＊IFA：incomplete Freund adjuvant（不完全フロインドアジュバント）

るが，その代表的なものを表1に示した。使用されている腫瘍抗原としては，やはりHLA-A24拘束性のCTL誘導性ペプチドが最も多い。

九大生医研外科のグループは，消化器癌においてもMAGE遺伝

子の発現が比較的高頻度に認められることを明らかにした。さらにHLA-A24拘束性のMAGE-3ペプチドの同定にも成功し，本邦で最も早く1997年1月にMAGE-3ペプチドとDCを用いた消化器癌に対するワクチン療法の臨床試験を開始した。彼らはワクチンを静脈内投与しているが，実際に腫瘍が縮小した大腸癌症例1例，食道癌症例2例を報告している[7]。われわれの施設においても1998年10月よりHLA-A24拘束性のCEAペプチド（CEA652）とDCを用いた消化器癌，肺癌に対する臨床試験を開始し，多くの免疫学的効果（腫瘍特異的細胞性免疫能の増強），臨床効果（腫瘍マーカーの低下，長期間の腫瘍増殖抑制）を確認した[8), 9)]。また国立癌センター中央病院では，米国DENDREON社製のクローズドシステムでのDCワクチン療法の臨床試験を，1999年から進行前立腺癌に対して開始している。成分採血〔apheresis〕で採取したDCを多く含む単核細胞分画に，40時間の行程でGM-CSFと前立腺性酸性ホスファターゼ（prostatic acid phosphatase：PAP）の融合蛋白を取り込ませた後に投与する方法であり，本邦での同システムの権利を有するキリンビール(株)医薬事業部からの委託研究という形で臨床試験を推進している。産官が一体となった今後の本邦における細胞免疫療法の臨床研究のモデルケースとして注目される。他方後章において詳述されるが，DCを用いない非細胞療法としての癌ワクチン療法の臨床試験も，久留米大学のグループを中心に精力的に進められている。彼らは，扁平上皮癌抗原（SART）を中心として，数多くの腫瘍抗原ペプチドを同定してきているが，これらをIFAと組み合わせて患者に投与している。最終的な製剤化を目指し，新薬の開発に類似したスタンスで臨床試験を展開している。

4．癌ワクチン療法の今後の方向性（表2）

科学的理論に裏打ちされた先進医療としての癌ワクチン療法は，臨床応用への道が開かれたばかりである。よって，倫理的観点からも，まず合成ペプチドやDCを用いた治療手法の安全性と免疫学的効果を検証することを主目的とし，従来の確立された治療法に不応性の高度進行癌症例を対象として，臨床試験が遂行されてきた。しかし，ワクチン療法本来の目的は感染症，癌を問わずあくまでその発症予防，あるいは微少残存病巣からの再発予防

表2 癌ワクチン療法の今後の方向性

A）微少残存病巣からの再発予防療法として（非細胞療法として）

1）ペプチドワクチン単独療法
2）低容量化学療法との併用
3）分子標的治療薬との併用

B）難治性癌に対する先進医療として（細胞療法あるいは非細胞療法として）

1）分子標的治療薬との併用
2）進化し続ける細胞療法（樹状細胞療法，造血幹細胞移植療法 etc.）との連動
3）新たに開発が期待される免疫抑制因子制御療法との連動

であり，免疫能が極端に荒廃した進行癌症例においては，能動免疫療法であるワクチン療法が有効に作用し得るはずのないことは，火を見るより明らかである[9]。よって，根治を目指した外科療法，放射線療法，化学療法後の微少残存病巣からの再発予防を目的とし，できるだけ多くの患者に還元可能な治療戦略を確立するための臨床試験を推進していくことが，今後最も重要であろう。製剤化を目指したペプチドワクチン単独療法，低用量化学療法や新たに開発，承認されてきている分子標的治療薬との併用療法が，重要な選択肢である。

他方，難治性進行癌に対する新たなるワクチン療法の開発も，今後に課せられた大きな命題の１つである。この領域においては，改良された樹状細胞療法や造血幹細胞移植療法との連動が必要不可欠であろう[10]。また，簡便で効果的な免疫抑制因子制御療法の確立が，ワクチン療法の飛躍的な効果増強のブレークスルーになりうる可能性がある。われわれの施設では現在，ポリスチレン系極細繊維で構成された免疫抑制物質吸着カラム（血漿置換を伴わない体外循環用治療カラム）を研究開発中である[11]。

おわりに

樹状細胞療法や同種免疫療法としての造血幹細胞移植療法の臨床導入，分子標的治療薬の目覚ましい勢いでの創薬，承認と，近年の癌治療の進歩は大変急峻である[12]。しかし，この進歩に医療現場のインフラ整備が追いついていないのが現状である。ようやく昨年，薬事法改正に基づく医師主導型臨床試験が可能となった

が，新しい癌治療技術の開発研究には産官学の協力，協調が必要不可欠である。ワクチン療法のみならず，多くの新しく開発される癌治療戦略を，日常臨床にいち早く還元できるような臨床試験システムを構築していくことが，今後の大きな課題であろう。

（上田祐二，山岸久一）

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Tumor HLA-DR expression linked to early intrahepatic recurrence of hepatocellular carcinoma

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The outcome of patients with hepatocellular carcinoma (HCC) remains poor because of the high frequency of intrahepatic recurrence (IHR), particularly early IHR within 1 year of hepatectomy. To search for genes involved in early IHR, we performed DNA microarray analysis in a training set of 33 HCCs and selected 46 genes linked to early IHR from approximately 6,000 genes by means of a supervised learning method. Gene selection was validated by a false discovery rate of 0.37%. The 46 genes included many immune response-related genes, which were all downregulated in HCCs with early IHR. Four of these genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*), encoding MHC class II antigens, were coordinately downregulated in HCCs with early IHR compared to levels in HCCs with nonrecurrence. A cluster analysis reproduced expression patterns of the 4 MHC class II genes in 27 blinded HCC samples. To localize the major site of production of HLA-DR protein in the tumor, we used 50 frozen specimens from 50 HCCs. Immunofluorescence staining showed that HLA-DR protein levels in tumor cells, but not in stromal cells, were associated with the transcription levels of *HLA-DRA* determined by both DNA microarray analysis and real-time quantitative reverse transcription-PCR. Univariate analysis showed that tumor HLA-DR protein expression, pTNM stage and venous invasion were associated with early IHR. Multivariate analysis showed that tumor HLA-DR protein expression was one of the independent risk factors for early IHR, suggesting HLA-DR protein potential as a biomarker and a molecular target for therapeutic intervention.
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Key words: HCC; microarray; MHC; HLA; recurrence

Hepatocellular carcinoma (HCC) is a common fatal cancer worldwide.[1–3] A major obstacle in the treatment of HCC is intrahepatic recurrence (IHR), which is observed in 30% to 50% of HCC patients who undergo curative surgery.[4,5] There are 2 representative modes of IHR after surgery, *i.e.*, early IHR and late IHR.[4,5] The former, most cases of which can be attributed to intrahepatic metastasis of cancer cells and detected within 1 year of surgery, limits the potential for surgical cure of HCC.[2,4,5] Thus, a better understanding of the molecular mechanisms of early IHR will enable us to develop novel therapeutic options for improving poor prognoses.

Since the initial description of DNA microarray analysis,[6] we have used this technology to profile gene expression patterns specific to many aspects of HCC.[5,7–10] Using a supervised learning method, we developed a DNA microarray-based system with only 12 genes for prediction of early IHR of HCC.[5] The 12-gene predictor detected accurately early IHR in HCC patients undergoing curative surgery; however, the 12 genes used are involved in a wide range of biological processes, and their roles in early IHR remain to be clarified.[5] To extend our previous findings,[5] we carried out a postplanned analysis of the DNA microarray datasets that were used to construct the 12-gene predictor.[5]

Our supervised learning procedure yielded 46 genes, which included many immune response-related genes in addition to the 12 genes mentioned above. Because 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the same MHC class II antigen family were included in 46 genes, we focused our present investigation on the identification of primary sites of HLA-DR protein biosynthesis in HCC tissues and examined the relation of protein levels to clinical features of HCCs.

Material and methods

Selection of genes related to early IHR by DNA microarray data analysis

We have analyzed the levels of expression of approximately 6,000 genes in human HCC samples using high-density oligonucleotide arrays (HuGeneFL Array, Affymetrix, Santa Clara, CA).[5,7–10] In our study, to search for genes related to early IHR, we used DNA microarray data from a sample of 33 HCCs, termed training sample, that was used to construct the predictor for early IHR (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/: released according to MIAME by Brazma *et al.*[11]). Among the 33 HCCs, early IHR was found in 12 HCCs (HCV02T, HCV06T, HBV07T, HBV14T, nonBC15T, HCV20T, HCV31T, HCV45T, HBV55T, HCV80T, HCV89T and HCV90T). No recurrence within 1 year after surgery was found in 21 HCCs (nonBC03T, HBV05T, HCV08T, nonBC09T, HCV10T, HCV12T, HCV18T, HCV21T, HCV22T, HCV26T, HCV27T, HCV28T, HCV29T, HBV30T, nonBC32T, HCV37T, HCV42T, HCV46T, HBV48T, HBV57T and HCV59T).

From a pool of approximately 6,000 genes, we first investigated all genes whose mean average differences (ADs) (arbitrary units from Affymetrix) in the 12 HCCs with early IHR were 2-fold higher or 0.5-fold lower than those measured in the 21 HCCs with nonrecurrence. We subsequently selected genes that had mean ADs >20 in either group. This filtering yielded 332 genes. We then ranked the selected genes in order of decreasing magnitude of Fisher ratios.[7–10] To decide how many genes we should consider, we used a random permutation test as described previously.[7–10] From the distribution of the Fisher ratios from randomized data, the top-ranking 46 genes with Fisher ratios >0.92 were considered to show statistically significant ($p < 0.01$) differences in expression between the 2 groups (Fig. 1 and Table I). To validate our gene selection, we calculated the false discovery rate, the percentage of false positive genes, as previously described.[10]

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FIGURE 1 – Gene expression profiles linked to early intrahepatic recurrence (IHR). Color displays of expression of 35 downregulated genes (upper panel) and 11 upregulated genes (lower panel) in HCCs with early IHR compared to HCCs with nonrecurrence. Each gene was ranked in decreasing order of the Fisher ratio (see Material and methods) and was listed with an accession number and symbol. Accession numbers for each gene were obtained from PubMed (http://www3.ncbi.nlm.nih.gov/PubMed/) or TIGR databases (http://www.tigr.org/tdb/hgi/searching/reports.html). Symbols were obtained from LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/).

To confirm reproducibility of the microarray data of 4 MHC class II genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*), we investigated their expression patterns in 27 blinded HCC samples (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/:) using hierarchical cluster analysis with Cluster software and Tree View software.[12]

TABLE I – FORTY-SIX GENES RELATED TO EARLY INTRAHEPATIC RECURRENCE OF HCC[1]

Fisher ratio	Accession no.	Symbol	Function	Locus
Thirty-five genes downregulated in HCC with early IHR *vs.* HCC without recurrence				
2.6879	L13923	FBN1	Extracellular matrix	15q21.1
2.5592	**L08895**	**MEF2C**	**Transcription**	**5q14**
1.9674	**M59465**	**TNFAIP3**	**Immune system**	**6q23.1–q25.3**
1.7562	**U51240**	**LAPTM5**	**Lysosomal protein interacting with ubiquity**	**1p34**
1.7390	M17733	TMSB4X	Immune system/cell shape and size control	Xq21.3–q22
1.6647	U69546	CUGBP2	RNA binding and RNA processing	10p13
1.5794	**X00274**	**HLA-DRA**	**Immune system**	**6p21.3**
1.5165	**X75042**	**REL**	**Transcription**	**2p13–p12**
1.5095	L43579	clone110298	Unknown	Xq28
1.3955	Z37976	LTBP2	Miscellaneous	14q24
1.3814	**X82200**	**TRIM22**	**Transcription**	**11p15**
1.3591	M33600	HLA-DRB1	Immune system	6p21.3
1.2798	U13219	FOXF1	Transcription	16q24
1.2626	M34996	HLA-DQA	Immune system	6p21.3
1.2569	U19495	SDF-1	Immune system	10q11.1
1.2163	**U59321**	**DDX17**	**Nuclear process or RNA binding protein**	**22q13.1**
1.2156	**Y10032**	**SGK**	**Signal transduction**	**6q23**
1.2130	**M21574**	**PDGFRA**	**Signal transduction**	**4q11–q13**
1.2028	M62424	F2R	Blood coagulation	5q13
1.1963	M13560	HLADG	Immune system	
1.1837	D28915	MTAP44	Immune system	1
1.1250	X82153	CTSK	Proteolysis and peptidolysis	1q21
1.1072	**Z19554**	**VIM**	**Cytoskelton**	**10p13**
1.0771	Z22534	ACVR1	Signal transduction	2q23–q24
1.0509	J03040	ON/SPARC	Ossification/extracellular matrix	5q31.3–q32
1.0354	M23178	SCYA3	Immune system	17q11–q21
1.0252	D13631	ARHGEF6	Apoptosis	Xq26
1.0195	**D13639**	**CCND2**	**Cell cycling**	**12p13**
0.9997	M55998	COL1A1	Extra cellular matrix	17q21.3–q22.1
0.9869	X64072	ITGB2/CD18	Immune system and cell adhesion	21q22.3
0.9858	Y00062	PTPRC	Miscellaneous	1q31–q32
0.9710	AB000409	MKNK1	Signal transduction	1pter–p31.3
0.9601	U20734	JUNB	Transcription	19p13.2
0.9364	Z84483	EST	Unknown function	13q12–q13
0.9202	U66075	GATA6	Transcription	18q11.1–q11.2
Eleven genes upregulated in HCC with early IHR *vs.* HCC without recurrence				
1.7441	U27326	FUT3	Metabolism	19p13.3
1.4150	L03411	RDBP	RNA binding and negative elongation factor	6p21.3
1.4096	X82693	E48	Cell adhesion	8q24-qter
1.3543	**AC000063**	**SEMA3F**	**Embryonic development**	**3p21.3**
1.3176	M20778	COL6A3	Extra cellular matrix	2q37
1.2370	J03060	GBAP	Metabolism	1q21
1.0222	M35531	FUT1	Metabolism	19p13.3
1.0018	U78190	GCHFR	Metabolism	15q15
0.9907	X13930	CYP2A	Detoxification and drug metabolism	19p13.2
0.9437	AF001359	MLH1	Mismatch repair	3p21.3
0.9263	U82306	EST	Unknown function	

[1]Bold face indicates the 12 genes used to construct the predictor.[5]

Immunofluorescence staining for HLA-DR protein

To identify the major location of HLA-DR protein in tumors, we used individual frozen specimens from 50 HCCs. All samples were immediately stored at −80°C in optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetechnical Co., Ltd., Tokyo, Japan) after surgery. Of these 50 HCC samples, 21 and 9 were assigned to the training and blinded sets, respectively, in the previous study.[5] The remaining 20 were newly recruited in our study. Direct immunofluorescence staining was performed as previously reported.[13] Briefly, 5 μm-thin cryostat sections were fixed in 95% ethanol. Sections were stained for HLA-DR and control sections were stained with hematoxylin and eosin. Individual sections were incubated with fluorescein isothiocyanate-conjugated murine monoclonal antibody (1:100) (Becton Dickinson Immunocytometry Systems, Mountain View, CA) against a nonpolymorphic human HLA-DR antigen overnight at 4°C. The immunoreactivity was analyzed with the use of Olympus BX50 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Positive HLA-DR immunoreactivity was found exclusively in kupffer cells and immune cells in nontumorous livers. Its positive frequency was less than 5% in all hepatocytes examined (data not shown). On the basis of this finding, tumor HLA-DR protein levels were scored as follows: 0, less than 5% of tumor cells were stained; 1, 5–30% of tumor cells were stained and 2, more than 30% of tumor cells were stained. Tumors scoring 1 or 2 were judged as overexpressed for HLA-DR protein. The scoring was performed independently by 2 pathologists blinded to clinical details. The scores assigned by the 2 pathologists were in agreement.

Real-time quantitative reverse transcription (RT)-PCR

To evaluate the relation between HLA-DR protein levels and *HLA-DRA* mRNA levels, we performed real-time quantitative RT-PCR in the 30 HCC samples mentioned above. Both the RT step and real-time quantitative PCR were performed as reported previously.[14,15] Standard plasmid (pAT153) containing the human *HLA-DRA* coding region was supplied by Health Science Research Resources Bank (Osaka, Japan). The plasmid was used as template DNA at numbers ranging from 10^3 to 10^8 copies to produce standard curves (data not shown). To detect human *HLA-DRA* mRNA, oligonucleotides 5′- CTCCCCTTCCTGCCCTCAAC-3′ (sense)

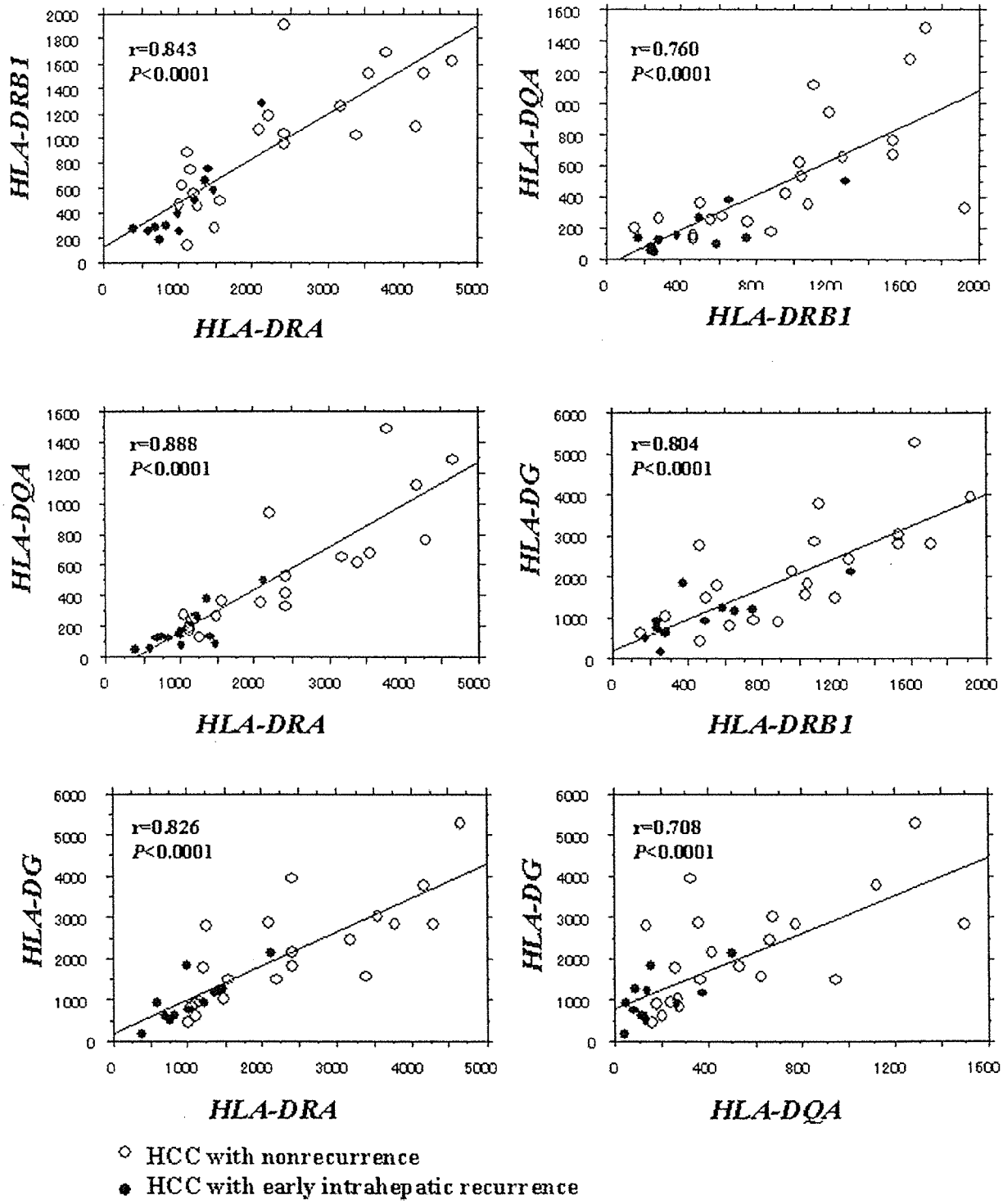

FIGURE 2 – Association among expression levels of 4 MHC class II genes determined by DNA microarray analysis. Pearson correlation coefficient was calculated using the software Statview. All values are average differences (arbitrary units from Affymetrix) of individual genes. All data are available at http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/. Note that all 4 genes are coordinately expressed in HCC tissues and expression levels are relatively low in HCCs with early intrahepatic recurrence.

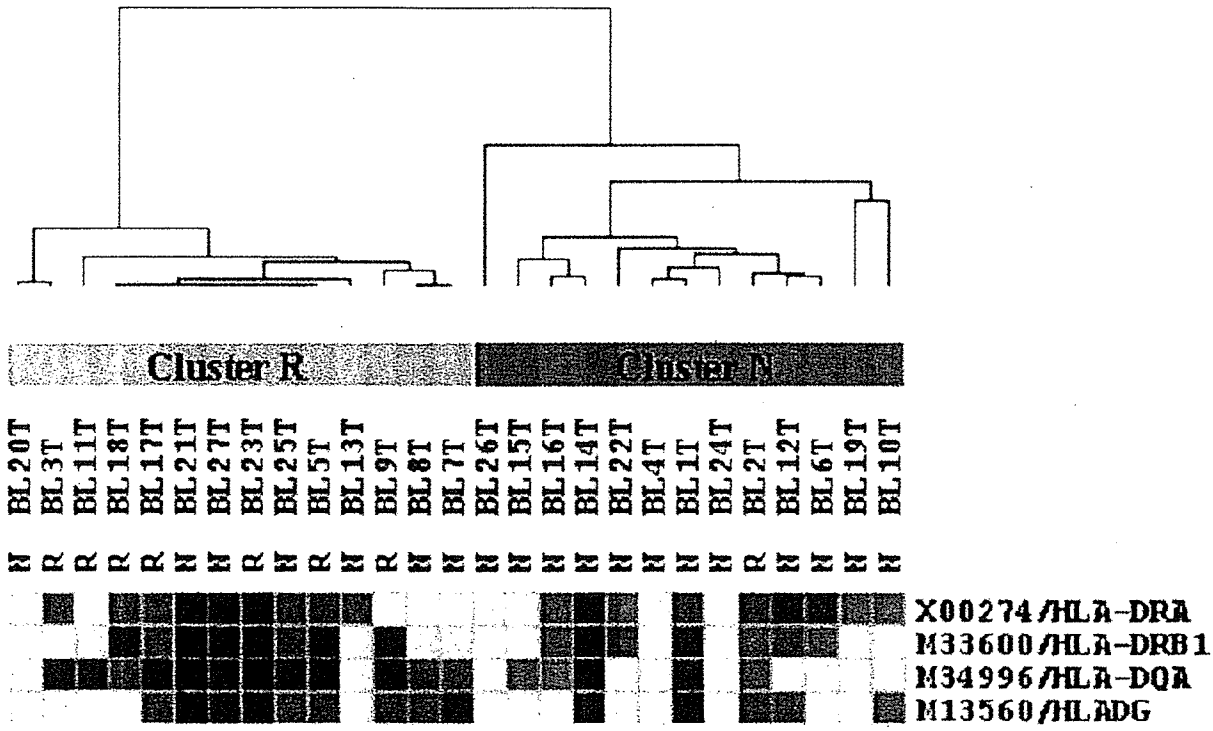

FIGURE 3 – Reproducibility of expression patterns of 4 MHC class II genes on an independent set of HCC samples. We investigated their expression patterns in 27 blinded HCC samples (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/:) using hierarchical cluster analysis with Cluster software and Tree View software.[12] R, HCC with early intrahepatic recurrence. N, HCC with nonrecurrence.

and 5′- ACCCACAGTCAGGCCCA AGG -3′ (antisense), which yield a 156 bp product were used as primers. Five microliters of cDNA solution (equivalent to cDNA from 100 ng of initial RNA) was subjected to real-time RT-PCR amplification. The reaction was performed on a LightCycler™ System Version 3 (Roche Diagnostics, Mannheim, Germany).[15] Products from PCR were quantified with a Lumi-Imager F1 (Roche Diagnostics) and analyzed with LightCycler Software (Roche Diagnostics). Each analysis was performed in duplicate. We calculated the abundance of each transcript as mean copy number per 100 ng RNA for each tissue.

Statistical analysis

Correlations between DNA microarray data of the 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) and correlations between DNA microarray data and quantitative RT-PCR data for the *HLA-DRA* gene were determined by Pearson correlation coefficients. Reproducibility of expression patterns of the 4 MHC class II genes on 27 blinded samples were validated by Fisher's exact test. Relations between HLA-DR protein and *HLA-DRA* mRNA levels in tumor were evaluated by ANOVA. Relations between tumor HLA-DR protein levels and clinicopathologic factors and relations between early IHR and clinicopathologic factors were analyzed by ANOVA with Fisher's PLSD test, Fisher's exact test and the Mann-Whitney U test. We carried out multivariate analysis to assess independent factors for early IHR in the 50 HCC samples using the stepwise logistic regression model (SPSS 11.0J; SPSS, Inc., Chicago, IL). Seven variables (age, sex, tumor HLA-DR protein levels, tumor size, tumor differentiation, venous invasion and pTNM stage) were entered into a forward stepwise regression model. Each model was tested for goodness of fit by -2 log likelihood and chi-square in each step. $p < 0.05$ was accepted as statistically significant.

Results

We identified 46 genes linked to early IHR of HCC. This gene selection was validated by a false discovery rate of 0.37%. Of those 46 genes, expression levels increased in 11 (23.9%) and decreased in 35 (76.1%) of HCCs with early IHR compared to levels in HCCs without early IHR (Table I). The latter group included many immune response-related genes (Fig. 1 and Table I) including 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the MHC class II antigen family whose expression levels were correlated (Fig. 2). Hierarchical cluster analysis with the 4 MHC-class II genes divided 27 blinded samples into 2 main clusters R and N. Seven of 14 had early IHR in cluster R; in contrast, 12 of 13 had nonrecurrence in cluster N (p=0.033 by Fisher's exact test) (Fig. 3). Thus, their expression patterns were maintained even in blinded HCC samples.

It is known that MHC-class I genes play a central role in host immune response in cancer patient.[16] There were many probes for MHC-class I genes or nonclassical genes on the array in our study; they did not survive in our filtering because their levels were markedly low in most HCCs examined (data not shown).

Among the 4 MHC class II family genes coordinately downregulated in HCC with early IHR, we chose to investigate the major location of HLA-DR protein in tumor. Using immunofluorescence staining, we found that HLA-DR protein was expressed in stromal cells in some cases, but its biosynthesis did not parallel the transcription levels determined by DNA microarray analysis (data not shown). For immunoreactivity of HLA-DR protein in tumor cells, 19 (38%) scored 0, 19 (38%) scored 1 and 12 (24%) scored 2 (Fig. 4). Levels of HLA-DR protein in tumor cells were associated positively with transcription levels of *HLA-DRA* gene by measured DNA microarray analysis and real-time quantitative RT-PCR ($p < 0.0001$ and $p = 0.0001$ by ANOVA) (Fig. 5). Tumor size in HCC scoring 0 was significantly larger than that in HCC scoring 1

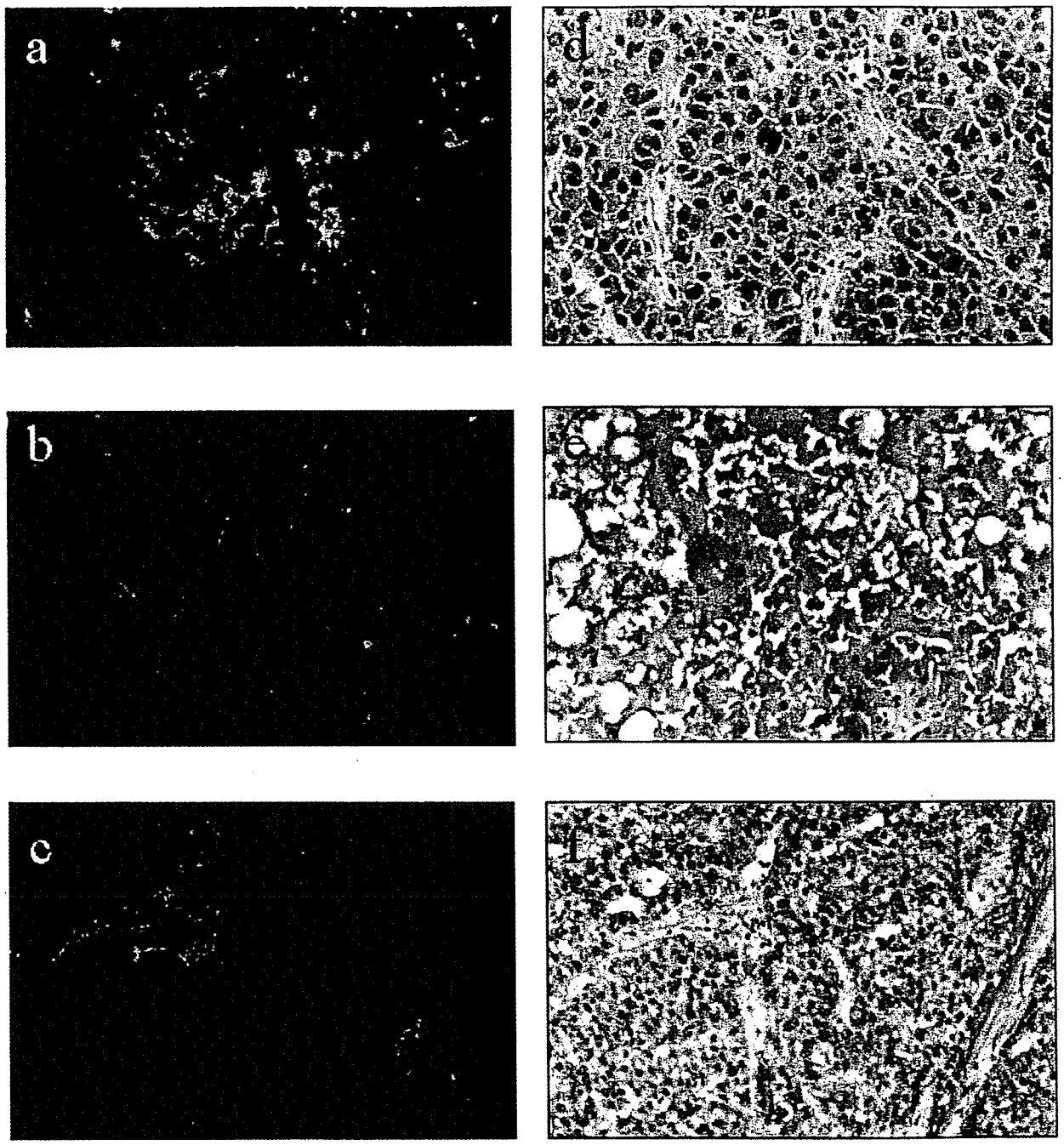

FIGURE 4 – Immunofluorescence staining for HLA-DR protein in representative cases. (*a*) A case (HCV10T) with score 2 in which more than 30% of tumor cells are stained. (*b*) A case (HCV45T) with score 1 in which about 10% of tumor cells are stained. (*c*) A case (HCV20T) with score 0. Note that some populations of stromal cells are stained, but none of the tumor cells are stained. All 3 tumors were judged as moderately differentiated HCC. (*d–f*) H&E staining of tumors corresponding to HCV10T, HCV45T and HCV20T, respectively (object lens: ×20).

FIGURE 5 – Levels of HLA-DR protein and *HLA-DRA* mRNA in HCCs. (*a*) Levels of HLA-DR protein and *HLA-DRA* mRNA in individual tumors. Note that there was an association between DNA microarray data and quantitative RT-PCR data in 30 samples ($r = 0.625$ and $p = 0.0001$). (*b*) DNA microarray data showing association between levels of HLA-DR protein and *HLA-DRA* mRNA in tumors ($p < 0.0001$ by ANOVA). (*c*) Quantitative RT-PCR showing association between levels of HLA-DR protein and *HLA-DRA* mRNA in tumors ($p = 0.0001$ by ANOVA). Score 0, less than 5% of tumor cells were stained; Score 1, 5–30% of tumor cells were stained; Score 2, more than 30% of tumor cells were stained. The RT-PCR result was based on mean of duplicated experiments in selected 30 samples.

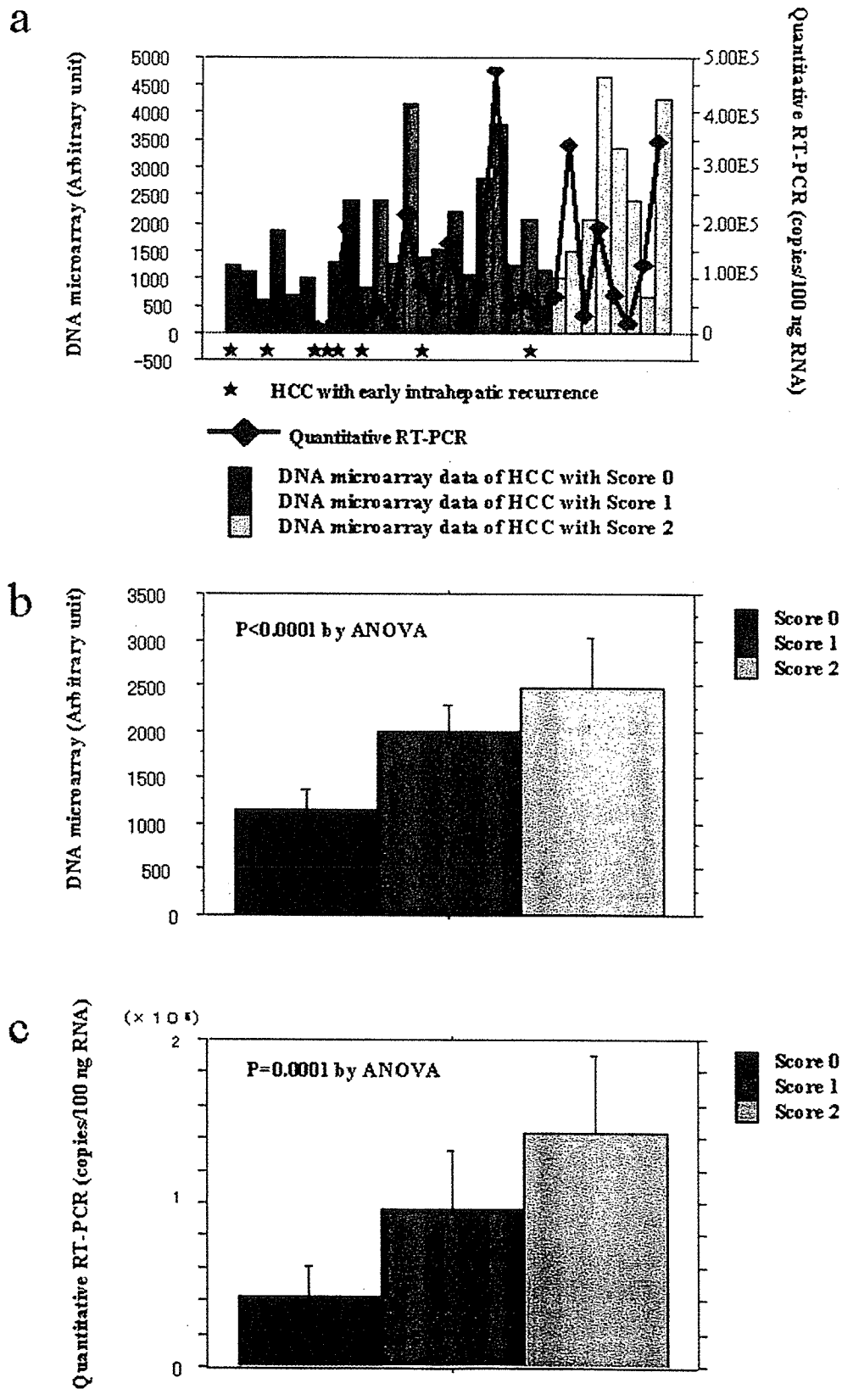
a
DNA microarray (Arbitrary unit)
Quantitative RT-PCR (copies/100 ng RNA)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
5.00E5
4.00E5
3.00E5
2.00E5
1.00E5
0
HCC with early intrahepatic recurrence
Quantitative RT-PCR
DNA microarray data of HCC with Score 0
DNA microarray data of HCC with Score 1
DNA microarray data of HCC with Score 2
b
DNA microarray (Arbitrary unit)
3500
3000
2500
2000
1500
1000
500
0
P<0.0001 by ANOVA
Score 0
Score 1
Score 2
c
Quantitative RT-PCR (copies/100 ng RNA)
(× 10⁵)
2
1
0
P=0.0001 by ANOVA
Score 0
Score 1
Score 2

FIGURE 5.

TABLE II – RELATIONS BETWEEN TUMOR HLA-DR PROTEIN LEVELS AND CLINICOPATHOLOGIC FACTORS

Clinicopathologic factors	Score of HLA-DR protein staining			*p* value
	0	1	2	
Sex				N.S.
Male	13	16	8	
Female	6	3	4	
Age (year)	61.9+/−2.4	63.5+/−1.2	64.0+/−2.3	N.S.
Tumor size (cm)	5.5+/−0.7*	3.2+/−0.4*	4.6+/−1.1	*$p = 0.015$
Tumor differentiation				N.S.
Well	0	2	0	
Moderately	17	12	11	
Poorly	2	5	1	
Venous invasion				N.S.
(−)	13	15	10	
(+)	6	4	2	
pTNM stage of UICC				**$p = 0.098$
I	5	11	8	
II	10	7	2	
IIIA	4	1	2	

*ANOVA with Fisher's PLSD test.–**Fisher's exact test, N.S., not significant.

TABLE III – FACTORS RELATED TO EARLY INTRAHEPATIC RECURRENCE

Factor	Early intrahepatic recurrence		*p* value
	(+) (n = 17)	(−) (n = 33)	
Sex			*$p = 0.334$
Male	14	23	
Female	3	10	
Age (year)	60.2+/−2.5	64.5+/−1.1	**$p = 0.120$
Tumor size (cm)	5.4+/−0.9	3.9+/−0.4	**$p = 0.167$
Tumor differentiation			*$p = 0.485$
Well	0	2	
Moderate	13	27	
Poor	4	4	
Venous invasion			*$p = 0.012$
(−)	9	29	
(+)	8	4	
pTNM stage of UICC			*$p = 0.038$
I	4	20	
II	9	10	
IIIA	4	3	
HLA-DR protein staining			*$p = 0.013$
score 0	11	8	
score 1	5	14	
score 2	1	11	

*Fisher's exact test.–**Mann-Whitney *U* test.

($p = 0.015$ by ANOVA with Fisher's PLSD test) (Table II). Low levels of tumor HLA-DR protein tended to be associated with advanced tumor stage ($p = 0.098$ by Fisher's exact test) (Table II). There were no associations between tumor HLA-DR protein levels and other clinicopathologic factors.

Univariate analysis showed that tumor HLA-DR protein levels, pTNM stage and venous invasion were associated with early IHR ($p = 0.013$, $p = 0038$ and $p = 0.012$) (Table III). There were no associations between early IHR and other clinicopathologic factors. Multivariate analysis showed that tumor HLA-DR protein levels and venous invasion were independent risk factors for early IHR (Table IV).

Discussion

We herein present the molecular signature linked to early IHR of HCC by applying a supervised learning method to DNA microarray technology. HCC patients have various backgrounds and divergent clinical courses, resulting in much heterogeneity among tumor samples examined.[8,17] To address this heterogeneity among patients, we applied the Fisher ratio and the random permutation test to DNA microarray data.[7–10] Thus, the 46 genes selected as described above represent the molecular signature specific to IHR in a larger number of HCC cases. In particular, when we compared the present data with our previous microarray data,[7,8,10] we found that there was no overlap between the 46 genes and virus- and liver cirrhosis-related genes. This means that the 46 genes are potential biomarkers and/or molecular targets for detection or treatment of early IHR in HCC and that the potential is independent of viral and nontumorous factors.

The 46 genes included 10 immune system-related genes, all of which were downregulated in HCCs with early IHR. This is one of the most striking findings of our present study. Several investigators including ourselves have proposed the clinical efficacy of immune therapy against HCC.[18–20] Therefore, our present result suggests that downregulation of host immune response plays a central role in early IHR of HCC. Four genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the same family of HLA class II antigens were coordinately downregulated in HCCs with early IHR in comparison to those with nonrecurrence. Their downregulation in HCC with early IHR was observed not only in training samples but also in blinded samples, indicating their predictive value for early IHR.

We focused our investigation on *HLA-DRA* of the 4 MHC-class II genes and showed for the first time that HLA-DR protein plays an important role in early IHR of HCCs. Several studies have

TABLE IV – INDEPENDENT RISK FACTORS FOR EARLY INTRAHEPATIC RECURRENCE

Variable	Regression coefficient	Standard error	Risk ratio (95%CI)	p value
Venous invasion				
(+)	2.981	1.169	19.699 (1.922–194.803)	$p = 0.011$
HLA-DR protein expression				
Score 1	−1.704	0.811	0.182 (0.037–0.893)	$p = 0.036$
Score 2	−3.689	1.558	0.025 (0.001–0.530)	$p = 0.018$
Sex				
Male	2.459	1.277	11.694 (0.957–142.848)	$p = 0.054$

shown that HLA-DR protein can be expressed by HCC cells; however, its relation to metastatic potential has not been discussed.[21,22] HLA-DR protein is involved in the antigen presenting function of macrophages including dendritic cells.[22] Activated lymphocytes also express the HLA-DR antigen.[23] Previous studies revealed the infiltration of HLA-DR-positive immune cells into HCC tissues following adoptive immune therapy.[24] Improved prognosis of HCC following infiltration by $CD8^+$ and $CD4^+$ T lymphocytes was found by Wada *et al.*[25] Because we did not perform laser capture microdissection, it is possible that the cancer samples tested contained stromal cells. In our previous microarray study,[5] we found that *vimentin* was preferentially produced by stromal cells in cancer tissues, and its downregulation was associated with early IHR of HCC. Taken together, these reported findings suggest that HLA-DR protein can be expressed in both tumor cells and stromal cells in HCC tissues. To identify the major site of HLA-DR protein biosynthesis, we carried out immunofluorescence staining of fresh tumor samples. Our present results showed that the HLA-DR protein was preferentially located in the cytoplasm of tumor cells, but not in that of stromal cells, and HLA-DR protein levels in cancer cells were consistent with *HLA-DRA* mRNA levels determined by quantitative RT-PCR and DNA microarray analysis. These results indicate that major sources of *HLA-DRA* mRNA and HLA-DR protein in tumor tissues are HCC cells themselves.

Multivariate analysis showed that low expression of HLA-DR protein in tumor is an independent risk factor for early IHR, suggesting its potential as a predictive marker for HCC with high metastatic potential. However, it remains unclear how tumor HLA-DR protein is related to intrahepatic metastasis of HCC. It is reasonable to assume the immunologic role considering that many immune-response related genes were downregulated in HCCs with early IHR as well as *HLA-DRA* and that no recurrence was found in 25 of 31 HCCs with HLA-DR overexpression (score 1 or 2). Many studies have showed that MHC-class II antigens play a role in progression of malignant tumors *via* immunologic modification.[26–31] Our current results were consistent with their results. In this regard, our finding is not a new one. However, in our study, it is noteworthy that the MHC-class II genes were selected without any bias from thousands of genes for association with early IHR in HCC. Interestingly, a microarray study by Ramaswamy *et al.*[32] showed similar finding that the decreased levels of *HLA-DP beta1* encoding 1 MHC class II antigen at the metastatic sites were commonly observed in a variety of adenocarcinomas. Recently, Herkel *et al.*[33] reported that MHC Class II-expressing hepatocytes can function as antigen-presenting cells and activate specific CD4 T lymphocytes. Epigenetic inactivation of the transcriptional activator for MHC class II genes is associated with the loss of HLA-DR expression in tumor cells.[34] Sartoris *et al.*[35] showed increased expression of HLA-DR in HCC cell lines by transfection of the transcriptional activator for MHC class II genes and found that the transfectant can serve to activate HLA-DR-restricted T cell line. In conjunction with these reports, our present results suggest immunologic roles of HLA-DR protein in early IHR of HCC. However, it should be noted that the HLA-DR protein was exclusively localized in the cytoplasm rather than on the cell membrane, raising the possibility that tumor HLA-DR has functions other than antigen-presenting function.[36] Further studies are needed to elucidate the biological function in early IHR of HCC.

In conclusion, our study shows that HLA-DR protein produced by tumor cells can be useful as a predictive marker for early IHR of HCC. Because the prognosis of HCC is extremely poor even when curative surgery is performed,[4,5] HLA-DR protein may also function as a molecular target to improve the poor prognosis.

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